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Subsets of Epidermal Langerhans Cells as Defined by Lectin Binding Profiles*

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In this study we characterize the cell surface glycoconjugate moieties of strain 2 guinea pig epidermal Langerhans cells (LC) in single cell suspension by using a battery of 17 fluorescent lectins. All LC displayed binding sites for concanavalin A, succinylated concanavalin A, *Lens culinaris* agglutinin, *Pisum sativum* agglutinin, wheat germ agglutinin, succinylated wheat germ agglutinin, *Griffonia simplicifolia* agglutinin I, *Ricinus communis* agglutinin I, *Phaseolus vulgaris* E agglutinin, and *Phaseolus vulgaris* L agglutinin, but failed to bind *So-*

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Abbreviations:

anti-Ia.2,4: strain 13 anti-strain 2 serum
EC: epidermal cell(s)
FITC: fluorescein-isothiocyanate
FITC-SpA: FITC-labeled staphylococcal protein A
HRP: horseradish peroxidase
Ia: immune response-associated (antigens)
LC: Langerhans cell(s)
Lectins:
Con A: concanavalin A
DBA: *Dolichos biflorus* agglutinin
GS I-B_4: *Griffonia simplicifolia* I-B_4 isolectin
GSL I: *Griffonia simplicifolia* agglutinin I
HPA: *Helix pomatia* agglutinin
LCA: *Lens culinaris* agglutinin
PHA-E: *Phaseolus vulgaris* E agglutinin
PHA-L: *Phaseolus vulgaris* L agglutinin
PNA: peanut agglutinin
PSA: *Pisum sativum* agglutinin
RCA I: *Ricinus communis* agglutinin I
SBA: soybean agglutinin
SJA: *Sophorica japonica* agglutinin
succ. Con A: Succinylated Con A
succ. WGA: succinylated WGA
UEA I: *Ulex europaeus* agglutinin I
WGA: wheat germ agglutinin
PBS-azide: phosphate-buffered saline supplemented with 0.02% sodium azide
TRITC: tetramethylrhodamine-isothiocyanate

***phora japonica* agglutinin (SJA), *Dolichos biflorus* agglutinin (DBA), and *Ulex europaeus* agglutinin I (UEA I). Neuraminidase pretreatment rendered LC reactive for SJA, but not for DBA and UEA I. The binding profiles of certain lectins point to the existence of LC subpopulations in that *Griffonia simplicifolia* I-B_4 isolectin, peanut agglutinin (PNA), *Helix pomatia* agglutinin, and soybean agglutinin bound to only 80% (range 70–90%) of Ia-positive epidermal cells; binding sites for these lectins on primarily unreactive Ia-positive cells were unmasked when epidermal cells were treated with neuraminidase prior to lectin labeling. Ultrastructural PNA labeling studies revealed that the vast majority of Birbeck granule-containing LC displayed PNA binding sites, whereas indeterminate cells were consistently PNA-negative. Identification of carbohydrate configurations expressed on LC surfaces by lectin binding may provide a clue for the elucidation of the mechanisms of established LC functions and possibly the discovery of as yet unknown properties of this cell type.**

It has been widely realized that all cells bear complex carbohydrate moieties at their surface attached both to proteins (forming glycoproteins) and to N-glycosphingosines (forming glycosphingolipids) [1]. Since cell surface carbohydrates play an important role in several specialized cellular functions including receptor activity, cell growth, differentiation, and interaction of cells with their environment [2,3], one may assume that the major biologic role for membrane carbohydrates is to serve as recognition molecules [2,3]. Whereas keratinocyte and melanocyte surface glycoconjugates have at least partly been characterized [4–22], no information at all exists on the surface carbohydrate composition of epidermal LC.

Lectins have emerged as a valuable tool for the investigation of the complex carbohydrates associated with cell surfaces [23,24] by their binding to specific sugar residues [25]. In this study we investigated the cell surface glycoconjugate pattern of guinea pig epidermal LC by using a battery of 17 lectins which recognize the majority of sugars usually present in membrane glycoproteins [26] and glycolipids [27].

MATERIALS AND METHODS

Animals

Initial breeding pairs of inbred strain 2 and strain 13 guinea pigs were obtained from breeding colonies of the National Institutes of Health, Bethesda, Maryland.

Antisera

The major histocompatibility complex of strain 2 guinea pigs differs from that of strain 13 guinea pigs only in the I-region [28]. Strain 13 anti-strain 2 (anti-Ia.2,4) serum was prepared as previously described [29]. Before use antiserum was inactivated at 56°C for 30 min.

Preparation of Epidermal Cell Suspensions

Single cell suspensions of strain 2 guinea pig epidermal cells (EC) were prepared from white-spotted abdominal skin by standard trypsinization procedures according to the method of Stingl et al [30].

Fluorescent Lectin Labeling

Reagents: TRITC-labeled lectins were purchased from Vector Laboratories (Burlingame, California), and E-Y Laboratories Inc. (San Mateo, California) and FITC-labeled GS I-B_4, PNA, SBA, and HPA from E-Y Laboratories Inc.; inhibitory sugars were obtained from Sigma Co. (St. Louis, Missouri).

Labeling Techniques:

(a) Fluorescent lectin staining reactions for visualization of cell surface glycoconjugates: Lectin labeling was carried out on unfixed cells to rule out possible fixation-induced alterations of cell surface glycoconjugates, in particular the exposure of masked lectin receptors. Artifacts possibly induced by redistribution phenomena and endocytic uptake of the lectins were prevented by performing incubation and rinsing procedures at 4°C with 0.02% sodium azide added to all solutions. Optimal working concentrations of labeled lectins were determined in pilot experiments as described previously [21]. For lectin labeling, 250 μl of precooled EC suspensions (resuspended to 4×10^6 EC/ml PBS-azide) were added to equal volumes of conjugated lectins diluted in PBS-azide to give a final concentration of 12.5–100 μg/ml (depending on the particular lectin); the reaction mixture was incubated at 4°C for 20 min, cells were then pelleted by centrifugation and washed 5 times with cold PBS-azide.

(b) Indirect immunofluorescence technique for identification of Ia-positive cells (i.e., LC): Ia-positive EC (i.e., LC) [30] were identified by an indirect immunofluorescence procedure using anti-Ia.2,4 serum and FITC-SpA as described previously [30]. Since GS I-B_4 isolectin, PNA, SBA, and HPA were available only as FITC conjugates, TRITC-labeled rabbit F $(ab')_2$ anti-guinea pig IgG diluted 1:20 with PBS-azide (Cappel Laboratories, Cochranville, Pennsylvania) was used instead of FITC-SpA for identification of Ia-positive cells.

Labeling Sequence: With each experiment separate samples of EC suspensions were subjected to: (1) fluorescent lectin labeling (= procedure *a*) followed by staining for Ia antigens (= procedure *b*) and to (2) Ia staining followed by lectin labeling. The strong reactivity of certain lectins (Con A, WGA, SBA, GSL I, RCA I, GS-I B_4) led frequently to agglutination of resuspended EC. To enable unequivocal evaluation of LC labeling profiles, EC were first attached (20 min, 4°C, PBS-azide) to poly-L-lysine-coated glass slides [31] and then labeled as described above. EC were used unfixed as well as prefixed (1% paraformaldehyde in PBS for 30 min at 4°C followed by exposure to 10 mM NH_4Cl in PBS for quenching of unreacted aldehyde groups) to check for possible redistribution phenomena of surface molecules following attachment.

Neuraminidase Pretreatment

For nonreactive lectins the binding capacity of LC was investigated after neuraminidase pretreatment. Unfixed EC were exposed for 60 min at 37°C to Vibrio comma neuraminidase (Behringwerke, Marburg, F.R.G.) at a concentration of 0.01 IU/ml in serum-free Medium 199, thoroughly rinsed with PBS, and then subjected to the labeling procedures as outlined above.

Control Experiments

Controls carried along with each experiment included: (1) incubation of cells with lectin conjugates in the presence of 0.1 M inhibitory sugar (see Table I) for determination of lectin binding specificity. In the case of PHA-E and PHA-L reacting with complex carbohydrate configurations [25], appropriate haptenic oligosaccharides were not available. (2) to rule out false negative lectin binding resulting from possible nonspecific action of the sugar on cell surface moieties (e.g., shedding of glycoconjugates), cells were also incubated in the presence of non-inhibitory sugar at 0.1 M concentration. (3) substitution of anti-Ia.2,4 serum by normal guinea pig serum as a negative control for the indirect immunofluorescence procedure detecting Ia-positive cells. (4) incubation of EC with TRITC-labeled lectins followed by FITC-SpA to exclude a possible affinity of FITC-SpA for lectin-treated cells; FITC-GS I-B_4 stained EC were incubated with TRITC rabbit F$(ab')_2$ anti-guinea pig IgG.

Ultrastructural Lectin Staining

Reagent: HRP-conjugated PNA was purchased from E-Y Laboratories Inc.

Labeling procedure: Determination of the optimal lectin concentration was performed as described in detail elsewhere [21]. Two hundred microliters of EC suspension (4×10^6/ml PBS) were added to equal volumes of HRP-conjugated PNA to give a final concentration of 200 μg/ml. The controls were incubated with the respective lectin in the presence of the specific inhibitory sugar (0.1 M). After incubating for 60 min on ice, cells were pelleted and washed 3 times with ice-cold PBS, fixed with precooled half-strength Karnovsky's fixative [32] for 60 min at 20°C, rinsed with 0.1 M cacodylate buffer (pH 7.4) followed by 0.05 M Tris-HCl buffer (pH 7.6), and subjected to the DAB reaction (20 min, 20°C in the dark) [33]. After rinsing procedures, cells were filtered onto Unipore polycarbonate membranes (pore size 0.2 μm, Biorad Laboratories, Richmond, California); to avoid cell losses the filters were then carefully covered with a 2% agarose solution, fixed for another 30 min in half-strength Karnovsky's fixative, and processed according to our standard embedding procedures [21]. Ultrathin sections were cut through the depth of the pellet, collected on hexagonal grids, and examined with a Philips EM 400 electron microscope operating at 80 kV.

Ultrastructural Identification of EC

Keratinocytes (K) were identified by bundles of tonofilaments and melanosome complexes. LC were identified by the established morphologic criteria, in particular by the presence of Birbeck granules [34]. Cells apparently lacking these organelles even on several consecutive sections but displaying all other morphologic criteria of LC were rated as "indeterminate" cells. Since these cells are known to express identical surface markers as Birbeck granule-containing LC, they are considered to belong to the LC lineage [35]. In order to ascertain that these indeterminate cells bear Ia antigens, a portion of the EC samples was processed for the immunoelectron microscopic demonstration of Ia antigens. EC suspensions (20×10^6/ml PBS-azide) were incubated for 30 min at 4°C with an equal amount of anti-Ia.2,4 serum (diluted 1:5), or with heat-inactivated serum from nonimmunized strain 13 guinea pigs for control purposes, washed 3 times, resuspended (5×10^6/ml PBS-azide), and reacted with an equal amount of protein A-iron-dextran (1 mg/ml) [36] for 30 min on ice, fixed in half-strength Karnovsky's fixative (60 min, 20°C) [32], and processed according to our routine embedding procedures [21].

RESULTS

General

Identical lectin binding patterns of LC were observed when lectin staining was either preceded or followed by Ia staining (see *Materials and Methods*). Similarly, attached EC and EC in suspension displayed identical lectin binding profiles, which were also not altered by prefixation of EC or by the presence of noncompeting sugars. All the other controls (see *Materials and Methods*) were consistently negative.

Lectin Binding Profiles of LC

According to their different binding patterns to LC, the lectins employed in this study can be divided into the following three categories (Table I):

Group 1: Lectins binding to all LC—Ten of the lectins reacted with all Ia-positive cells (i.e., LC) in a given suspension (Table I). These lectins reacted also with the majority of other EC but with varying degrees of intensity.

Group 2: Lectins unreactive with LC—Three of the lectins used conformed to this binding pattern (Table I). Whereas UEA I did not bind to any EC even after neuraminidase pretreatment (but reacted strongly with human O erythrocytes used as a positive control), DBA and SJA reacted with a considerable portion of EC, but did not bind to any LC. Neuraminidase pretreatment was performed in order to determine whether this lack of reactivity was due to either an absence or an inaccessibility of the respective sugar residues. Such a treatment failed to uncover DBA binding sites, but rendered LC reactive for SJA (Table I). These results indicate a true absence of DBA binding sites and suggest that SJA receptors are naturally masked by sialic acid on LC.

Group 3: Lectins binding to subfractions of LC—Four lectins conformed to this binding pattern in that GS I-B_4, PNA, SBA, and HPA bound only to approximately 80% (range 70–90%) of Ia-positive EC (Table I). LC reactive with PNA, SBA, and HPA displayed uniform fluorescence intensity, whereas among GS I-B_4 positive LC, we observed an inverse relationship between cell size and lectin staining intensity (Table I). Ia-positive EC unreactive with either GS I-B_4, PNA, SBA, or HPA in general were smaller than those possessing binding sites for these lectins (Figs 1, 2). Neuraminidase pretreatment induced binding sites for all group 3 lectins on initially unreactive LC; unmasking of the respective receptors was apparent with the lectins SBA, HPA, and PNA, but less obvious with GS I-B_4.

Ultrastructural PNA labeling revealed important differences between LC and indeterminate cells. Whereas the majority of Birbeck granule-containing LC displayed PNA binding sites, indeterminate cells (i.e., cells exhibiting all morphologic criteria of LC but apparently devoid of Birbeck granules) were consistently unreactive for PNA (Fig 3). Both cell populations, however, were uniformly Ia-positive as demonstrated by immunoelectron microscopy (not shown).

DISCUSSION

LC are dendritic epidermal cells and play a functional role in the afferent limb of the immune response which is similar to that of Ia-bearing macrophages [37]. Since it is very likely that—analogous to macrophages [38]—many of the functional capacities of this important cell type [39] depend on cell surface

TABLE I. *Lectin labeling profiles*

Lectins	Percentage of lectin binding EC		Percentage of lectin binding LC	
	Untreated	Neuraminidase	Untreated	Neuraminidase
Group 1				
Con A	100 (+++)[a]	n.d.[b]	100 (+++)	n.d.
succ. Con A	100 (+)	n.d.	100 (+)	n.d.
LCA	95 (+/++)	n.d.	100 (++)	n.d.
PSA	95 (+/++)	n.d.	100 (++)	n.d.
WGA	99 (++/+++)	n.d.	100 (++)	n.d.
succ. WGA	78 (+)	n.d.	100 (+)	n.d.
GSL I	95 (++/+++)	n.d.	100 (++)	n.d.
RCA I	99 (++/+++)	n.d.	100 (++)	n.d.
PHA-E	100 (++/+++)	n.d.	100 (++)	n.d.
PHA-L	100 (++/+++)	n.d.	100 (++)	n.d.
Group 2				
SJA	55 (+)	100 (++/+++)	0	100 (++)
DBA	25 (+)	40 (++)	0	0
UEA I	0	0	0	0
Group 3				
GSI-B_4	90 (+/++/+++)	100 (++/+++)	75–90 (+/++)	100 (++/+++)
PNA	94 (+/++)	100 (++/+++)	70–90 (+)	100 (++)
HPA	25 (+)	100 (++)	70–90 (+)	100 (++)
SBA	85 (+/++)	97 (++/+++)	65–85 (+)	100 (++)

[a] Fluorescence intensity: expressed semiquantitively using a scale range from − to +++.

[b] n.d. = not determined.

Major sugar specificities [25]: *Con A, succ. Con A, LCA, PSA*: α-D-mannosyl, α-D-glucosyl-; *WGA*: (β-N-acetylglucosaminyl-)n, sialic acid; *succ. WGA*: β-N-acetylglucosaminyl-)n; *GSL I*: α-D-galactosyl and N-acetyl-α-D galactosaminyl end groups; *RCA I*: β-D-galactosyl-; *PHA-E, PHA-L*: complex carbohydrate binding specificity; *SJA*: N-acetyl-β-D-galactosaminyl-; *SBA, DBA, HPA*: N-acetyl-α-D-galactosaminyl-; *UEA I*: α-L-fucosyl-; *GSI-B_4*: α-D-galactosyl end groups; *PNA*: β-D-galactosyl-(terminal).

Inhibitory sugars (0.1 M): *Con A, succ. Con A*: α-methyl-D-glucopyranoside; *LCA, PSA*: α-methyl-D-mannopyranoside; *WGA, succ. WGA*: N′N′-diacetyl chitobiose; *GSL I*: α-D-meliobiose; *RCA I*: lactose; *PHA-E, PHA-L*: no inhibitory sugar available; *SJA, DBA, HPA, SBA*: N-acetyl-D-galactopyranoside; *UEA I*: L-fucose; GSI-B_4, *PNA*: α-methyl-D-galactopyranoside.

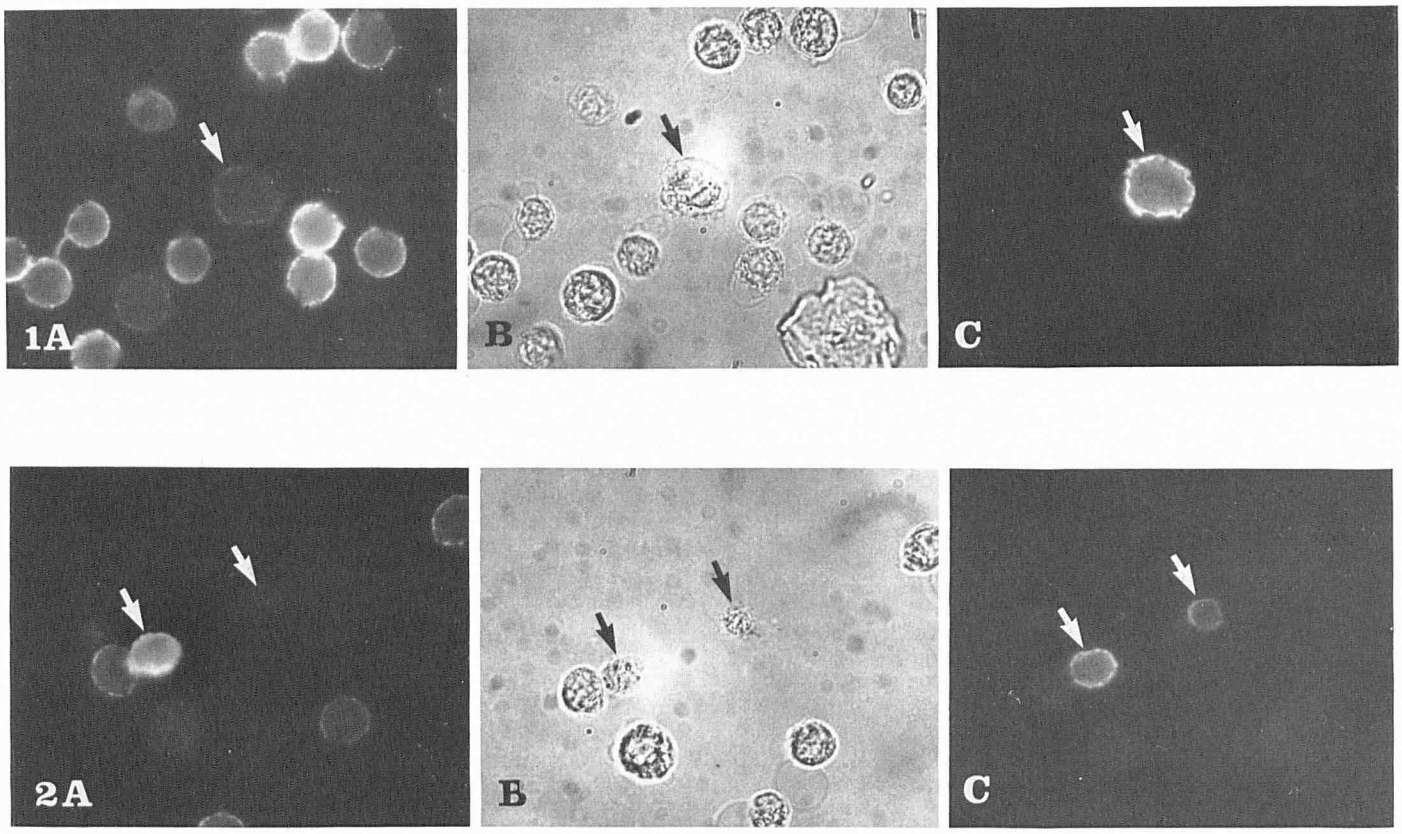

FIG 1 and 2. EC suspensions were labeled with FITC-conjugated GS I-B_4 lectin followed by Ia staining (indirect immunofluorescence technique employing TRITC-labeled second antibody) for identification of LC. A particular field was photographed by plain light microscopy (*B*), FITC (*A*), and TRITC (*C*) filter setting. × 800. *Fig 1: Arrows* denote LC (*C*) only slightly reactive with FITC GS I-B_4 (*A*). *Fig 2: Arrows* denote a small and a medium sized LC unreactive and strongly reactive for FITC GS I-B_4 lectin (*A*), respectively.

interactions, we have been interested in defining the complex surface carbohydrate moieties by using lectins as specific probes. We elected to study EC suspensions rather than frozen skin sections for several reasons: first, identification of LC and their surface glycoconjugates is easier; secondly, interpretation is more accurate due to the absence of the constituents of the extracellular space; and thirdly, all in vitro studies on the functional capabilities of LC have also employed this model system.

The binding of group 1 lectins to LC surfaces is not surprising since the respective carbohydrate residues commonly are found on most cell types so far investigated, including keratinocytes and melanocytes [1,4–22].

Neuraminidase pretreatment of EC showed that the failure of certain lectins (group 2) to bind to Ia-positive EC may have different reasons. Since neuraminidase failed to uncover UEA I and DBA receptors on LC, one may suggest that the respective binding sites are either absent or present only in such minute amounts that they escape detection by the methods used; alternatively, it is conceivable that these residues are sterically inaccessible due to glycosylation of neighboring sugar residues [25]. In contrast, neuraminidase pretreatment of EC enables LC to bind SJA, which indicates that the respective receptor sites are present, but normally masked by sialic acid—a phenomenon by no means unique to LC [21,22,40–44].

By far the most interesting finding arising from the present work is the existence of lectins (group 3) which react only with certain LC subsets. The additional finding that neuraminidase pretreatment restored the capacity of LC to bind all group 3 lectins suggests that cell membranes of LC differ in their degree of sialylation.

Though we got the impression that LC unreactive with GS I-B_4, PNA, SBA, and HPA were not only smaller but generally also less Ia-positive than LC reactive with these lectins, flow cytofluorometric studies are required to confirm these observations. Double-labeling experiments at the ultrastructural level are needed to determine to what extent group 3 lectins identify overlapping LC populations.

A striking observation was that indeterminate cells consistently lacked PNA binding sites whereas the majority of Birbeck granule-containing LC proved to be PNA reactive. Whether these differences in lectin binding profiles reflect the existence of functionally diverse LC subsets (including indeterminate cells) is as yet unknown but appears conceivable in that differentiation and maturation [42,45,46], activation [47], homing mechanisms [48], receptor function [49], and immune recognition [50] of immunoactive cells are critically linked to the quantity and quality of sugar moieties expressed on their cell membrane. Although according to current understanding, cells of the LC lineage are the only Ia-positive cells within normal guinea pig epidermis [30,35,39], one should even consider the theoretical possibility that a minor portion of the Ia-positive EC (e.g., those that fail to bind group 3 lectins) might represent a hitherto unrecognized, functionally distinct EC population. In order to determine whether subfractions of Ia-positive EC as defined by different lectin binding patterns differ in their capacity to induce antigen-specific and/or allogeneic T-cell activation [37], we are currently using group 3 lectins for the fractionation [51] of highly enriched LC suspensions [52].

In summary, identification of carbohydrate configurations expressed on LC surfaces by lectin binding has revealed the existence of LC subpopulations and may provide a clue for the

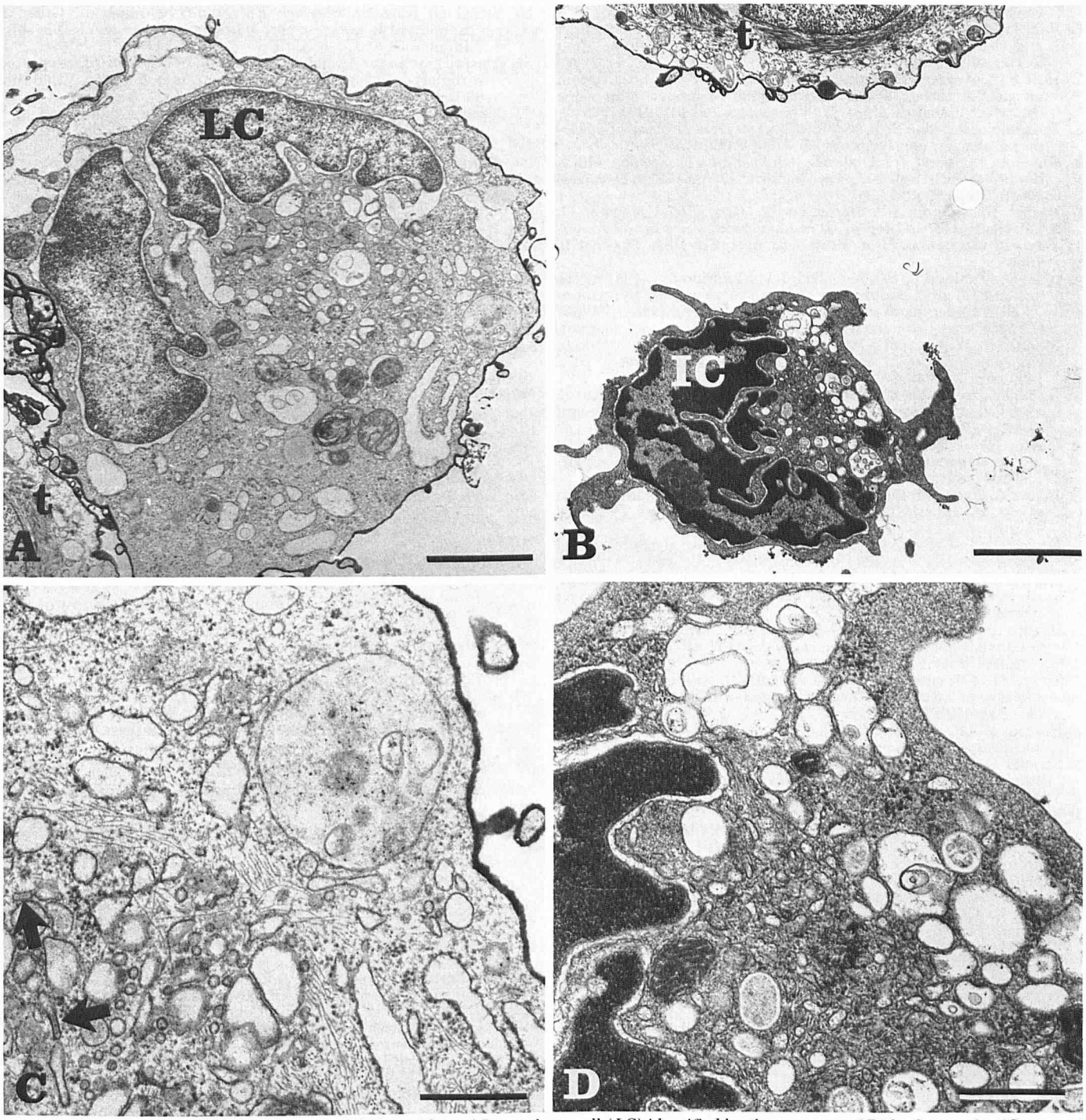

FIG 3. Ultrastructural PNA labeling. *A and C*, An epidermal Langerhans cell (*LC*) identified by the presence of Birbeck granules (*C, arrows*), as well as an adjacent keratinocyte (*t* = tonofilaments) display PNA binding sites on their cell membranes as evidenced by deposition of HRP reaction product (*A, C*). *B and D*, No PNA reactivity is disclosed on an indeterminate cell (*IC*) (*B, D*), whereas an adjacent keratinocyte posesses PNA binding sites as indicated by deposition of HRP reaction product. *Bars* = 2 μ (*A, B*) and 0.5 μ (*C, D*).

elucidation of the mechanisms of established LC functions and possibly the discovery of as yet unknown properties of this cell type.

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